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A SUBMERSION METHOD FOR THE CULTURE OF HYDROGEN-OXIDIZING BACTERIA: GROWTH-PHYSIOLOGICAL INVESTIGATIONS

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H.G.Schlegel, H.Kaltwasser, and G.Gottschalk*

A method for submersion culture of knallgas bacteria is described, in which the nutrient solution is agitated magnetically under a mixture of H_2, O_2, and CO_2 and the high O_2-sensitivity of the cells is allowed for by "gradient gassing". The facultative chemolithotrophic Hydrogenomonas strain 20 was bacteriologically characterized and growth-physiologically investigated. During the logarithmic phase, the generation time was 2 1/6 hrs, and the apparent doubling time was 3 1/5 hrs (28°C).

The scarcity of investigations on the growth physiology of aerobic hydrogen-oxidizing bacteria (knallgas bacteria) is primarily due to the lack of a simple reliable method for cultivation under optimum conditions. Development of a method for the submersion culture of knallgas bacteria, under autotrophic conditions, is made difficult by the fact that three necessary components of the nutrient substrate (molecular hydrogen, carbon dioxide, and oxygen) must be supplied to the organisms in the gaseous state and the fact that this gas mixture is explosive.

In most investigations to date, knallgas bacteria were either grown on agar (Kluyver and Manten 1942; Schatz and Bovell 1952; Atkinson and McFadden

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** Numbers in the margin indicate pagination in the original foreign text.

1954; Atkinson 1955, 1956; Packer and Vishniac 1955; Marino and Clifton 1955) or as pellicles on liquid nutrients (Kaserer 1906; Niklewski 1908; Lebedeff 1909; Ruhland 1922, 1924; Schlegel 1953, 1954). In cultivating on the surface of agar or of stagnating nutrient solutions, relatively thick layers or pellicles will form; the cells are exposed to varying conditions, and the harvested material contains a mixture of bacteria of different physiological age. Uniform material, which can be coordinated with a certain growth phase, cannot be obtained by this method. In addition, the surface technique is very laborious and requires unphysiologically long incubation times; after an incubation of 4 - 5 days of 150 inoculated agar plates, 2.5 gm cells (dry weight) were harvested (Packer and Vishniac 1955).

A considerable improvement was obtained by growing knallgas bacteria in fluid cultures, by shaking under an oxyhydrogen atmosphere (Bergmann, Towne, and Burris 1958; Linday and Syrett 1958); however, application of this method is restricted to small quantities.

Bubbling the CO_2-oxyhydrogen mixture through the inoculated mineral solution in a closed system (Wilson, Stout, Powelson, and Koffler 1953; Cohen and Burris 1955; Bergmann, Towne, and Burris 1958) obviously creates optimum conditions for growth of knallgas bacteria, so that the method can be recommended for growing of mass cultures. However, the method has the disadvantage that the foaming, observed in forced gassing, must be suppressed by the addition of an antifoaming agent. A feasible but technically difficult method would be /210 pumping the inoculated nutrient solution through a column packed with glass spheres and bubbled through with the gas mixture (McFadden 1959).

The most important prerequisite for optimum growth of knallgas bacteria in homogeneous suspension is saturation of the liquid phase with the gas components.

In our laboratory, a submersion method for the autotrophic culture of knallgas bacteria has been highly successful, in which distribution of the gas mixture throughout the mineral solution is obtained by strong magnetic agitation. The technical expenditure is low. During intense stirring, the cell growth progresses logarithmically up to 1 gm dry weight/ltr nutrient solution.

1. Methods

Turbidimetry was performed as absorption measurement in 1-cm cuvettes at 436 mμ in an Eppendorf Photometer. The measurements were made only within the extinction region between 0.1 and 0.3. The protein content of the suspension was determined by the Weichselbaum (1946) biuret method, modified by La Rivière (1958) for bacteria.

The number of cells was determined by counting in a Thomas counter (according to Neubauer). The total nitrogen was determined according to Kjeldahl. The dry weight was obtained after twice washing the cells in distilled water and drying at 85° C up to weight constancy. The pH of the nutrient solutions and buffers was adjusted under control with the glass electrode and the pH meter of the Methrom AG. The respiratory metabolism was measured with conventional two-neck vessels of about 15 ml volume, in the round Warburg apparatus (Model V by the Braun Co., Melsungen); 116 shakings/min; 4 cm shaking amplitude.

Nutrient solution. The mineral medium contained, per 1000 ml dist. water: 9 gm $NaHPO_4 \cdot 12\ H_2O$; 1.5 gm KH_2PO_4; 1.0 gm NH_4Cl; 0.2 gm $MgSO_4 \cdot 7\ H_2O$; 1.2 mg $FeNH_4$ citrate; 20 mg $CaCl_2$; 2 ml Hoagland solution; 0.5 gm $NaHCO_3$ (separately sterilized). The pH was 6.8 - 7.0. For heterotrophic culture, the bicarbonate was replaced by 2 gm glutamate.

Organism. The investigated organism (Hydrogenomonas strain 20) is a gram-

negative nonsporulating motile short rod. The cells are 1.5 - 1.7 μ long and 0.8 - 1.0 μ wide. In liquid nutrients, the cells separate readily and form homogeneous suspensions, without lumping; even in stagnating solution, no pellicle formation takes place.

On mineral agar plates, the organism grows in the form of round slightly cambered glistening yellow colonies with a smooth edge. In heterotrophic culture (glutamate), the colonies merely differ from the above by their white color. The growth on glucose is weak.

The organism is characterized by still other biochemical properties. It grows on meat broth without pellicle formation and on potato slants in the form of yellowish-brown colonies; on blood agar, the organism does not produce hemolysis, utilizes citrate and a number of organic acids, reduces nitrate to nitrite, forms no indole, no hydrogen sulfide, no methylethylcarbinol, does not liquify gelatin, and does not alkalize or coagulate milk.

Culture vessels and stirring apparatus. As culture vessels (Figs.1 and 2), 6-ltr two-neck flasks of Solidex (Pyrex) glass were used. Ordinarily, these are filled with 3 - 4 ltr mineral medium. For supplying and removing the gas, a washing bottle attachment, provided with two absorbent cotton filters and inserted into the central neck of the flask with a NS 29, is used. The /211 second flask neck (NS 29) is provided with a heavy glass tube, closed on top with a ground stopper and extending into the nutrient solution; through this glass tube, samples can be taken with sterile pipettes without escape of the gas. A bar magnet made of oerstite 400-Fe, of 15 mm diameter and 60 mm length watertight welded into the thick-walled polyvinyl chloride tube, is placed into the nutrient solution. Both flask and nutrient are sterilized in the autoclave; the rod magnet is stored in 80% ethanol and sterilized by rinsing with sterile

water.

The gas mixture (75% H_2, 15% O_2, 10% CO_2) is produced in a gasometer connected with an equalizing vessel; tap water, acidified with H_2SO_4, is used as sealing liquid. A washing bottle, between the gasometer and the culture vessel, prevents possible leakage of the sealing liquid into the culture flask.

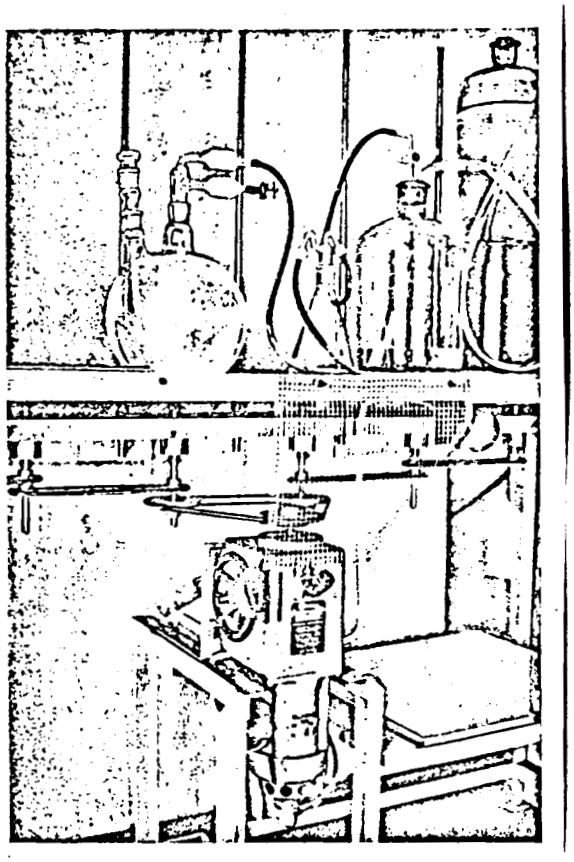

Fig.1 Apparatus for Submersion Culture of Knallgas Bacteria under Autotrophic Conditions.

The apparatus (Fig.1) is equipped with six stirring devices. The drive magnets (oerstite prong magnets, width 5 cm), rotating in cut-outs of the table top, are driven over a transmission by an 0.5-hp three-phase motor with continuously variable all-steel gearing (by the Heynau Co., Munich). The equipment was built by the local firms E.Schütt Jr. and H.Clodius. /212

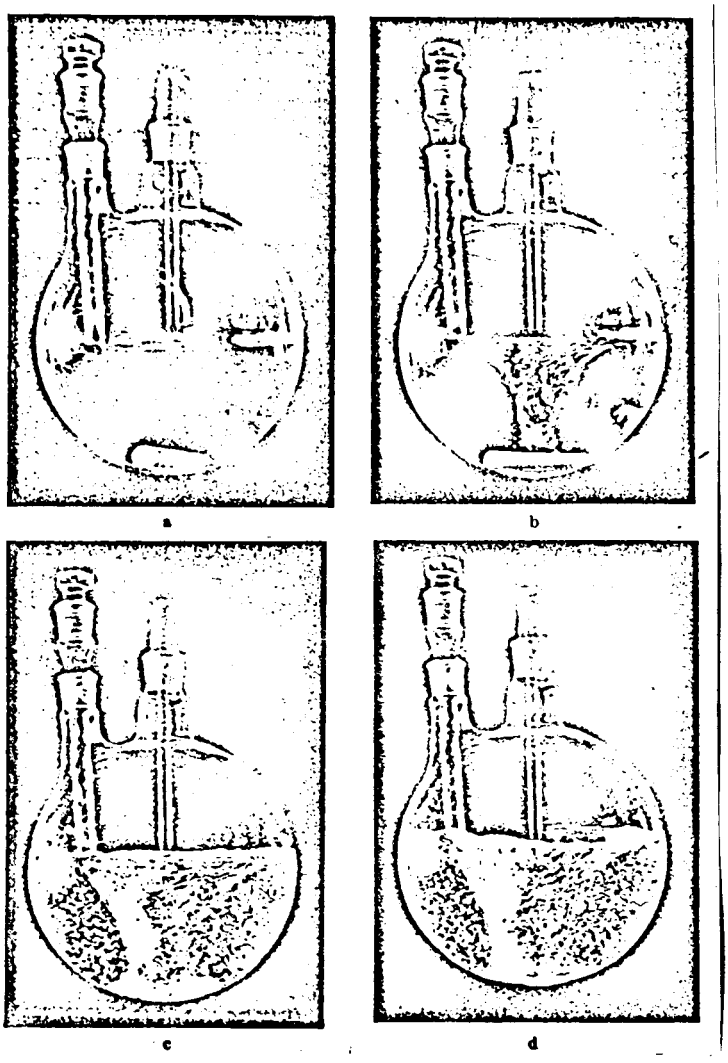

Fig.2 Influence of the Rotational Speed of the Magnetic Stirrer on the Gas Distribution in the Nutrient Solution. Rpm: a = 0; b = 375; c = 650; d = 800.

After inoculating the nutrient solution, a 10-ltr gas mixture is bubbled through the gas volume of the culture vessel; the gas space remains connected with the gasometer over a tube; the gas drain at the culture flask is closed off. Figures 2a - d show the distribution of the gas in the mineral solution, produced by the stirring action, at various rotational speeds. Our laboratory routine is to grow knallgas bacteria at 650 rpm.

2. Results

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a) Growth Rate Curve

For determining the growth rate curve, a 6-ltr flask (3 ltr mineral solution) was inoculated with cells taken from a mineral agar plate which had been incubated autotrophically for four days; every 2 hrs, 4 ltr of a gas mixture

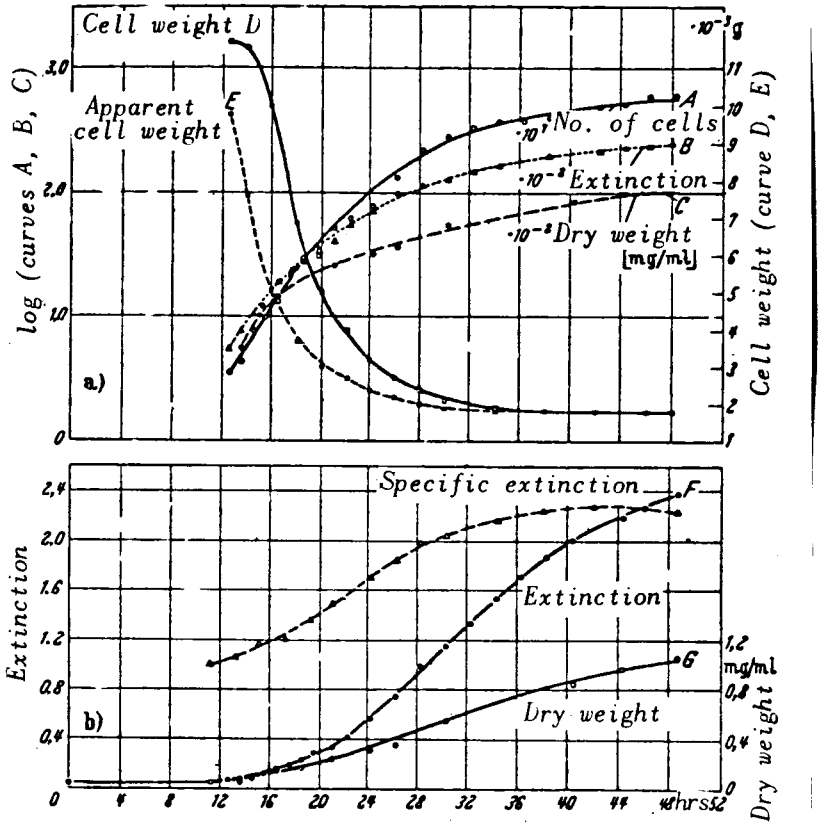

Fig.3a and b

a - Change in number of cells (A), extinction (B), dry weight (C), cell weight (D), and apparent cell weight (E) during autotrophic growth (A - C, semilogarithmic); b - Increase in dry weight, extinction, and specific extinction (extinction per milligram dry weight). 6-ltr two-neck flasks with 3 ltr mineral solution of pH 7.0; inoculation with 4-day old cells, grown on agar plates; 28°C; 650 rpm; gas mixture: 85% H_2, 10% O_2, 5% CO_2.

Fig.3a upper right, read 10^{-13} gm instead of 10^{-3} gm.

of 85% H_2, 10% O_2, and 5% CO_2 was bubbled through the flasks. This gassing, repeated within short intervals, was found necessary since H_2 and O_2 are con-

verted by the bacteria at an approximate ratio of 3:1 and since the atmosphere will thus become depleted in O_2. Turbidity and total number of cells were determined from the very beginning, while dry weight, biuret and Kjeldahl /214 nitrogen were determined only during the logarithmic growth phase. Figure 3a (in semilogarithmic presentation) shows the increase in total number of cells (curve A), turbidity (curve B), and dry weight (curve C) during a 50-hour growth experiment. Under the given conditions - with only 10% O_2 in the gas atmosphere - the phase of the exponential fission rate is relatively short; already after 20 hours, a decrease in this rate takes place obviously due to a limitation of the oxygen diffusion. The slope of the curves for both dry weight and turbidity is less. In curve D, the quotient dry weight/total number of cells = = cell weight is plotted. At the beginning of the logarithmic fission phase, the cells reach their maximum dry substance content. During the logarithmic period, the cell weight first decreases rapidly and then more slowly. It is obvious that the cell dimensions vary constantly ever during the log phase, which means that the cells do not represent "standard cells" (Henrici 1923; Martin 1932; Hershey 1939). This is a circumstance that must be taken into consideration in physiological investigations on cells that are harvested at a certain instant of time.

In curve E, the quotient "apparent dry weight"* /number of cells = "apparent cell weight" (Hershey 1939) is plotted. The "apparent cell weight" differs considerably from the real weight. The deviation is due to the dependence of the light scattering on the size of the scattering particles. A comparison of the curve G (dry weights) in Fig.3b with the curve F (extinction values) demon-

* The "apparent dry weight" is strictly proportional to the extinction and can be calculated from the extinction values and from a once and for all determined factor (extinction/dry weight at the end of the test).

strates that the ratio of extinction/dry weight = "specific extinction" increases from the beginning of the logarithmic phase; with a decrease in cell weight, i.e., in particle size, the specific extinction increases.

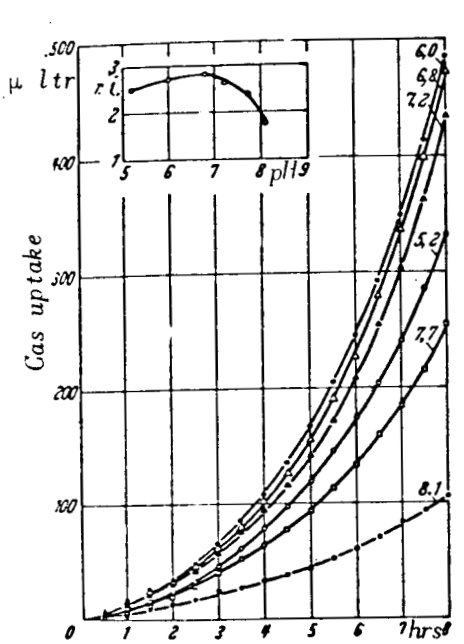

Fig.4

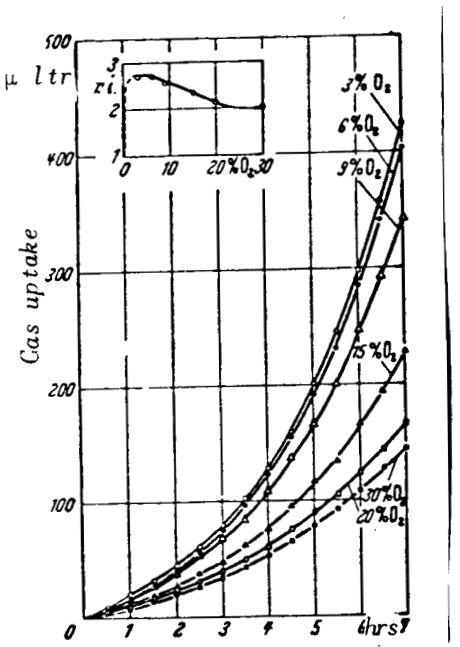

Fig.5

Fig.4 Gas Uptake under Growth Conditions at Varying pH. In the experiment, 3 ml mineral medium were inoculated per Warburg vessel with 0.005 mg cells (dry weight); 30° C; gas atmosphere: 30% air, 65% H_2, 5% CO_2; the accurate pH of the mineral solutions buffered with phosphate was determined with the glass electrode after saturation with the gas mixture. For plotting the pH optimum curve, the relative increase in gas uptake between the third and sixth hour of experiment was plotted:

$$\text{r.i.} = \frac{\mu\text{ltr gas uptake after 6 hrs} - \mu\text{ltr gas uptake after 3 hrs}}{\mu\text{ltr gas uptake after 3 hrs}}$$

Fig.5 Gas Uptake under Growth Conditions at Varying pO_2. Used were 3 ml mineral medium per Warburg vessel, inoculated with 0.008 mg cells (dry weight); 30° C; pH 6.5; gas atmosphere 30% H_2, 5% CO_2, 3 - 30% O_2, filled to 100% with N_2. Optimum curve as in Fig.4.

b) Determination of Growth Constants by Respiratory Metabolism Measurements

The optimum growth conditions (pH, temperature, pCO_2, pO_2, pH_2) were

determined by measuring the gas absorption of a very thin suspension (0.005 - - 0.015 mg cell dry weight) over a period of 8 hrs. Cells, pipetted off the logarithmic phase of the submersion culture, increase in the Warburg vessels without lag phase. Since the gas absorption of log-phase cells can be set proportional to the increase in cell mass, the growth can be checked from the increase in gas uptake. The optimum curves (Figs.4 - 6) are based on the relative increase (r.i.) of gas uptake between the third and sixth hour of the experiment (see legend in Fig.4).

The strain 20 grows in a relatively wide pH range (Fig.4). The growth /215 optimum is located between pH 6.5 and 7.0. Shifts in pH toward the acid end from the neutral point influence the growth less than shifts toward the alkaline end.

Figure 5 shows the great sensitivity of the investigated strain to elevated oxygen partial pressures. At an oxygen content of the atmosphere of 30%, growth just about takes place; above 40%, the growth is suppressed, as had been observed at various occasions (Schatz and Bovell 1952; Schlegel 1953). At the lowest tested O_2 partial pressure (3% O_2), the rate of growth is greatest. It should be noted that the measurements were made on very thin suspensions which caused a just about discernible turbidity. Under these conditions, the dissolved /216 gases are in equilibrium with the gas atmosphere, and the pO_2 in the solution corresponds to that in the gas volume. Consequently, the optimum curve reproduces the "true" dependence of the cell growth on the oxygen partial pressure. However, if the growth, at higher cell concentrations, is limited by the oxygen diffusion rate, higher O_2 partial pressures will actually have a promoting effect.

The temperature optimum of the growth is 32°C (Fig.6). At higher tempera-

tures (35° and 40°C), the gas uptake first will be greater because of the higher reaction velocity of the enzymes present; however, from the decreasing tendency in gas uptake it can be concluded that no cell increase occurs and that the enzyme system is damaged in fact.

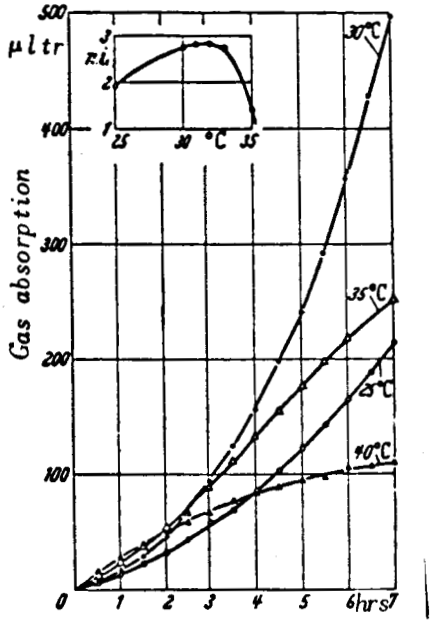

Fig.6 Gas Uptake under Growth Conditions at Varying Temperature.
Per Warburg vessel, 3 ml mineral medium inoculated with 0.015 mg cells (dry weight); pH 6.5; gas atmosphere: 65% H_2, 30% air, 5% O_2; optimum curve as in Fig.4.

The growth is practically independent of the CO_2 partial pressure in the range of 1 - 10%; at higher CO_2 contents, the gas uptake is impaired. In how far the growth, at different H^+ ion concentrations, is influenced by different CO_2 partial pressures, has never been investigated.

In the range of 3 - 80% H_2, the growth is almost independent of the H_2 partial pressure. At still lower H_2 concentrations, we did not check on the growth but instead determined the hydrogenase activity. Half the maximum velocity of H_2 oxidation is reached at 0.007 atm.

c) Dependence of the Growth on the Oxygen Partial Pressure, at Various Cell Densities

In determining the "true" dependence of the growth of knallgas bacteria (strain 20), the greatest rate of growth was observed at low hydrogen concentrations (3% O_2). Under the conditions used and at extremely low bacteria concentration, complete equilibrium in the sense of the Henry-Dalton law existed /217

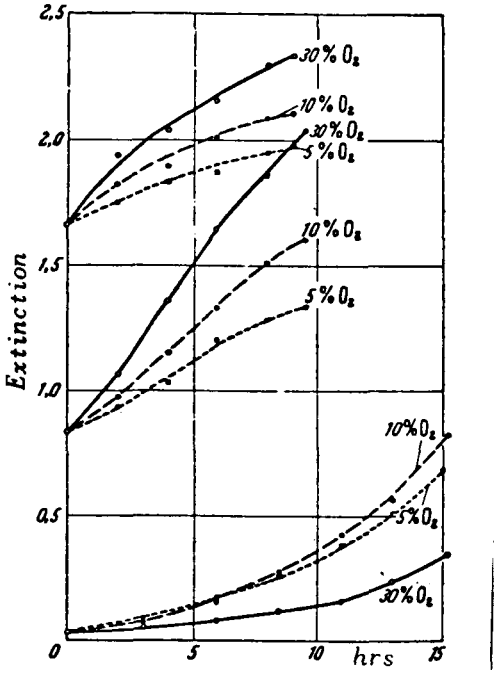

Fig.7 Dependence of the Growth on pO_2, at Various Cell Concentrations.
3 ltr mineral medium of pH 6.8 in 6-ltr two-neck flasks at 28°C, rotational speed 650 rpm; inoculated from a fluid preliminary culture; gas atmosphere: 5, 10, and 30% O_2 + 10% CO_2 + H_2 ad 100%; turbidity measurement.

between the pO_2 in the gas atmosphere and in the suspension liquid. At greater bacteria densities, as they usually occur during the growth of a culture, different conditions prevail: Absorption of the dissolved oxygen by the organisms takes place more rapidly than can be brought into solution by O_2 diffusion from the gas phase. Even on enlarging the phase interfaces by fine division of the

gas in the liquid (by stirring, shaking, or bubbling), no oxygen saturation of the solution will occur. In a nutrient solution, visibly rendered turbid by actively oxygen-consuming microorganisms, the O_2 partial pressure will always be lower than in the adjacent gas atmosphere.

The growth-accelerating effect of higher O_2 partial pressures on the cells, in an advanced stage of the culture, had been observed already in investigations on the strain 16 (Wilde, unpublished). In Fig.7, the result of an experiment is plotted in which nutrient solutions, inoculated to various degrees, were incubated under gas mixtures of differing O_2 content. The growth was checked by turbidity measurements. At a low original cell density, a high O_2 partial pressure (0.3 atm) will inhibit the growth; the most rapid increase in turbidity takes place first at 5% and later at 10% O_2 in the gas mixture. The upward slope of the curves indicates an exponential growth in this particular range. At a higher initial cell concentration (U = 0.83), the rectilinearity of the rise in turbidity indicates that the cells grow arithmetically, meaning that the rate of growth is limited by a constantly acting factor; the rise angles, increasing with increasing oxygen content of the gas mixture (5, 10, 30% O_2), indicate that the limiting factor here is the oxygen diffusion rate. Based on still higher cell densities (U = 1.66), the growth rate curves even show declining tendencies; the growth, in this range, is no longer proportional to /218 the gas uptake.

For mass cultivation of knallgas bacteria, their "true" oxygen sensitivity is without significance as soon as their oxygen consumption, at progressing growth, leads to an inequilibrium of the O_2 partial pressures of gas and liquid phase. Under the selected growth conditions (650 rpm; 28°C; 3 - 4 ltr mineral solution in 6-ltr flasks), the culture developed fastest if a gas mixture with

at first 10% O_2 and, after reaching a bacteria concentration of U = 0.5 - 0.8, with 30% O_2 was used.

The use of a 30% O_2-containing gas mixture facilitates monitoring the culture, since this mixture contains the components approximately at the volumetric ratio at which the gases are absorbed during the growth process ($H_2:O_2:CO_2$ = 5.8:1.8:1, according to Marino and Clifton 1955). This eliminates the gassing which had been necessary at short intervals. At a suitable arrangement of series-connected gas reservoirs, filled with different gas mixtures - i.e., using "gradient gassing" - the culture can be left to itself until the stationary phase is reached.

3. Discussion

Knallgas bacteria grow in a simple mineral medium, containing NH_4Cl as nitrogen source and trace elements as well as iron. Despite the fact that no detailed investigations as to the dependence of the growth on the composition of the mineral solution are in existence, certain observations indicate that knallgas bacteria tolerate concentration fluctuations of the individual components within a wide range. Conversely, the composition of the gas atmosphere and all factors that control the transport of the gas components to the cells have a decisive influence on the rate of growth. Since carbon dioxide is rapidly absorbed by aqueous solutions, it always is available in the cells in sufficient amounts. Even molecular hydrogen, because of its small molecular volume and its high rate of diffusion, reaches the site of consumption sufficiently rapidly; in our experiments (pH_2 > 0.1 atm), the H_2 diffusion never was a limiting factor. Of all factors, influencing the growth in submersion cultures, oxygen has the greatest significance. This reduces the question of gas supply in knallgas

bacteria to the problem of oxygen supply, which plays a decisive role in the submersion culture of all intensely oxygen-consuming strictly aerophilic micro-organisms (Rahn and Richardson 1941, 1942; van Niel 1949).

Only at extremely low cell densities and as long as turbidity is hardly /219 perceptible to the eye does the oxygen dissolved in the medium correspond to the O_2 partial pressure in the gas phase, in the sense of the Henry-Dalton law. With increasing O_2 consumption of the organisms, an increase in O_2 deficit in the solution occurs, until finally the growth is inhibited by O_2 deficiency. At higher cell densities, the rate of O_2-supply rather than the rate of growth is measured. The duration of the phase of exponential growth can be used as a criterion for the efficiency of oxygen supply.

A prolonged and adequate oxygen supply is obtained in our method by brisk mechanical stirring and by using an oxygen-rich gas mixture. This method, which can be safely used for a large number of aerophilic organisms is rendered more difficult in the case of knallgas bacteria because of their O_2-sensitivity, in so far as high O_2 partial pressures inhibit the growth at low bacteria densities (Figs.5 and 7). We had mentioned previously that this distinct O_2-sensitivity can be compensated in part by a sort of "gradient gassing", under gradual increase in O_2-content from 5 to 30%.

Figure 3 indicates that the increase in dry weight during the growth cannot be checked by turbidity measurements. It has become routine to draw conclusions as to the cell mass from the turbidity, which is measured as extinction or "optical density". Already Hershey (1939) concluded from experiments on Escherichia coli that "it can be seen that the ratio of turbidity to nitrogen is constant within the expected error in spite of the threefold difference in average size of cells in the two suspensions. The nephelometric method is,

therefore, suitable for the estimation of bacterial mass in cultures of different age ...". It is even possible to calculate the generation time from values obtained by absorption measurements (Cohen and Burris 1955). However, these methods are useful only in rough approximation for a representation of the real conditions.

During the growth of a stationary culture, the cells undergo considerable changes in size. These size changes are accompanied by a change in the relative surface of the cells and in the "specific" extinction (related to the dry weight or to the cell mass).

Incubation Time hrs	Dry Weight mg/ml	Extinction	Specific Extinction
15	0.1	0.12	1.20
30	0.55	1.12	2.04
45	0.99	2.26	2.28

An increase in cell size corresponds to a decrease in "specific extinction". This specific extinction, shown in Fig.3b and increasing with decreasing cell size, which also is confirmed by records published by Hershey (1939), agrees with Mie's theory on the scattering of light. The scattering is dependent on the particle size and on the number of particles. Consequently, turbidity measurements - no matter whether they are done turbidimetrically or nephelometrically - cannot yield conclusions as to the cell mass unless it can be proved that the particles, within the investigated range, remain constant in size and in other properties determining the scattering of light. /220

With our submersion method and using the strain 20, a relatively high rate of growth was measured. From the log phases of the growth rate curves (Fig.3), a generation time of 2 hrs 10 min was computed (from the increase in cell concentration) and an "apparent" doubling time of 3 hrs 12 min (from the increase

in extinction), at 28° C. For Hydrogenomonas facilis in submersion culture, a doubling time (at 30° C) of 7.5 hrs (Cohen and Burris 1955) and 5 hrs (Bergmann, Towne, and Burris 1958) was reported. The apparent doubling time, designated by the authors as generation time, was calculated from the extinction data. However, our own measurements clearly indicated that a difference must be made between "generation time" and "doubling time". For the gas absorption, a doubling time of only $1\frac{1}{2}$ - $1\frac{3}{4}$ hr is obtained under optimum conditions (Figs.4 and 6).

For further investigations on hydrogen-oxidizing bacteria, the described procedure most likely will be the method of choice. The method offers the best basis for the development of a continuous culture process. Because of the technical simplicity and the ready cultivatability of knallgas bacteria, this method is useful also for the biosynthesis of C^{14}-labeled compounds (Newton and Wilson 1954) from carbon dioxide. The method is less laborious than other preparation methods described for this particular purpose (Erb and Maurer 1960).

4. Summary

A method is described for the submersion culture of knallgas bacteria. The principle of the process is a brisk magnetic stirring of the nutrient solution, under a mixture of H_2, O_2, and CO_2. The high O_2 sensitivity of the cells is allowed for by a "gradient gassing".

The facultative chemolithotrophic Hydrogenomonas strain 20 was bacteriologically characterized and growth-physiologically investigated. During the /221 logarithmic phase, the generation time is 2 1/6 hrs and the apparent doubling time, 3 1/5 hrs (28° C).

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assistance.

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